08-24-00 A

08/23/00 Jc883 U.S. PTO

UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional applications under 37 CFR 1.53(b))		
	Title of Invention	Methods of Obtaining 2-Methoxyestradiol of High Purity
	Named Inventor(s)	Gregory E. Agoston, Jamshed H. Shah, Anthony M. Treston
	Attorney Docket	05213-0541
	Express Mail Label No.	EL561432388US

APPLICATION ELEMENTS

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

1. ☐ Fee Transmittal Form
   (Submit an original, and a duplicate for fee processing)
2. ☒ Specification, Claims, and Abstract — Total Pages 30
3. ☒ Drawings — Total Sheets 16
4. Oath or Declaration — Total Pages 0
   a. ☐ Newly executed (original or copy)
   b. ☐ Copy from prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17 completed)*
   [Note Box 5 Below]
   (i) ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation by Reference
   (usable if Box 4b is checked)
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Microfiche Computer Program *(Appendix)*
7. ☐ Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. ☐ Computer Readable Copy
   b. ☐ Paper Copy (identical to computer copy)
   c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment:
   a. ☐ Assignment Papers (cover sheet & document(s))
   b. ☐ Assignment is of record in parent application No. ______
9. ☐ 37 CFR 3.73(b) Statement
   (when there is an assignee)
   ☐ Power of Attorney by assignee
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS) PTO-1449
    ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
    (Should be specifically itemized)
14. ☐ Small Entity Statement(s)
    ☐ Statement filed in prior application Status still proper and desired
15. ☐ Certified Copy of Priority Document(s)
16. ☐ Other: ______

Jc880 U.S. PTO 09/644387 08/23/00

17. If a **CONTINUING APPLICATION**, *check appropriate box and supply the requisite information*:
    ☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No:
    Recite complete dependency back to first parent application: ______

18. CORRESPONDENCE ADDRESS:

Kimberly J. Prior, Esq.
JONES & ASKEW, LLP
2400 Monarch Tower
3424 Peachtree Road, N.E.
Atlanta, Georgia 30326

By: Kimberly J. Prior Reg. No. 41,483
Date: August 23, 2000
Telephone: 404-949-2400
Facsimile: 404-949-2499

09644387 .082300

METHODS OF OBTAINING 2-METHOXYESTRADIOL OF HIGH PURITY

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 60/150,293 filed August 23, 1999.

FIELD OF THE INVENTION

The invention relates to the estradiol metabolite 2-methoxyestradiol and to methods of obtaining purified 2-methoxyestradiol.

BACKGROUND OF THE INVENTION

2-Methoxyestradiol, 1,3,5(10)-estratrien-2,3,17β-triol-2-methyl-ether (2-ME2) is an endogenous metabolite of estradiol, the major ovarian estrogen. The chemical formula of 2-ME2 is $C_{19}H_{26}O_3$, and the compound has a molecular weight of 302.4. 2-ME2 has low of estrogenic activity but has been found to have other biological effects.

U.S. Patent Nos. 5,504,074, 5,661,143, and 5,892,069 to D'Amato *et al.* disclose methods of treating mammalian diseases characterized by abnormal cell mitosis using 2-ME2. Undesirable cell mitosis is characteristic of many diseases, including, but not limited to, cancer, atherosclerosis, proliferation of solid tumors, vascular malfunctions, endometriosis, retinopathies, arthropathies, and abnormal wound healing. In addition, cell mitosis is important in a wide variety of biological functions, including but not limited to the normal development of the embryo, formation of the corpus luteum,

cyclic proliferation of uterine endometrium, wound healing, and inflammatory and immune responses.

U.S. Patent No. 5,521,168 to Clark discloses using 2-ME2 for lowering intraocular pressure. 2-ME2 also inhibits estrogen-induced pituitary tumor angiogenesis and suppresses tumor growth in Fisher 344 rats as reported by Banerjee, S.K. *et al.*, Proc. Amer. Assoc. Cancer Res. 39, March 1998.

Presently, commercially available preparations of 2-ME2 are either less than 98% pure or contain undesirable steroid contaminants that are of concern for pharmaceutical uses. Important contaminants of these preparations are estradiol, 4-hydroxyestradiol, 4-methoxyestradiol, 2-hydroxyestradiol, estrone, and 2-methoxyestrone. The amounts of these contaminants that are found in presently available 2-ME2 preparations are unacceptable for pharmaceutical applications.

Any therapeutic use of 2-ME2 in humans requires 2-ME2 having a high level of purity. In general, therapeutic agents are required to be substantially pure to avoid negative side effects of contaminants. In particular, since 2-ME2 has effects that are counteracted by estradiol and other estrogenic metabolites, it is crucial to have a 2-ME2 preparation substantially free of such contaminants. Effects that may be seen from contaminating estradiol, estrone, and 2-hydroxyestradiol include estrogenic effects such as feminization, endometrial proliferation, increased risk of uterine and breast cancer, developmental effects on sexual organs, inhibition of leukopoesis, and effects on hematopoetic cells. 4-hydroxyestradiol, 4-methoxyestradiol, and estradiol are known mutagens and carcinogens.

Accordingly, what is needed is a composition of 2-ME2 which is greater than 98% pure and which contains substantially no estradiol or other steroids having estrogenic or carcinogenic effects.

What is also needed is a composition containing 2-ME2 that is greater than 99.5% pure.

What is also needed are methods for making 2-ME2 of greater than 98% purity and containing substantially no estradiol or other steroids having estrogenic or carcinogenic effects.

Also needed are methods of substantially separating 2-ME2 from estradiol, related molecules, and other contaminants, resulting in 2-ME2 having a purity of greater than 99.5%.

SUMMARY OF THE INVENTION

The present invention provides 2-ME2 having greater than 98% purity, more preferably greater than 99% purity, most preferably greater than 99.5% purity. The 2-ME2 preparations preferably contain less than 0.03% estradiol, 0.02% or less 2-hydroxyestradiol, 0.02% or less 4-hydroxyestradiol, 0.02% or less 4-methoxyestradiol, and less than 0.02% estrone. More preferably, the 2-ME2 preparations contain 0.01% or less estradiol, 0.02% or less 2-hydroxyestradiol, 0.01% or less 4-hydroxyestradiol, 0.01% or less 4-methoxyestradiol, and 0.01% or less estrone.

The present invention also provides methods of obtaining 2-ME2 of greater than 98% purity, more preferably greater than 99% purity, most preferably greater than 99.5% purity. In some embodiments, the methods involve synthetic techniques. In other

embodiments, the methods involve purification techniques to separate the 2-ME2 from other compounds. In yet other embodiments, the methods involve both synthetic techniques and purification techniques described herein.

The purification methods involve the use of liquid-solid chromatography (LSC) to separate 2-ME2 from other compounds. The chromatographic media is preferably silica. The solvent system comprises a non-polar solvent, such as chloroform, and a polar solvent, such as methanol.

Accordingly, an object of the present invention is to provide 2-ME2 having a purity greater than 98%.

Another object of the present invention is to provide 2-ME2 substantially free of estradiol, related compounds, and other unwanted impurities.

Still another object of the invention is to provide methods of obtaining substantially pure 2-ME2 by synthetic techniques.

Another object of the invention is to provide methods of obtaining substantially pure 2-ME2 by purification techniques.

Other features and advantages of the invention will be apparent from the following description of preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chromatogram from the reversed phase HPLC analysis of 2-methoxyestradiol available from Sigma Chemical

Company (45H4033). This graph shows that the Sigma product contains about 0.034% estradiol.

Figure 2 is an expanded view of the chromatogram in Figure 1 indicating the estradiol impurity.

Figure 3 is a chromatogram from the reversed phase HPLC analysis of 2-methoxyestradiol available from Research Plus (10699). This graph shows that the Research Plus product contains about 0.024% estrone and about 0.93% other undesirable estrogens.

Figure 4 is an expanded view of the chromatogram in Figure 3 indicating the estrone impurity.

Figure 5 is a chromatogram from the reversed phase HPLC analysis of the unpurified 2-methoxyestradiol employed as the starting material in Example 2 of the present invention.

Figure 6 is a chromatogram of the 2-ME2 of the present invention produced in Example 2. The HPLC was run with a non-overloaded amount of sample, 75.6 µg (14 µl at 5.4 µl/ml).

Figure 7 is an expanded view of the chromatogram in Figure 6.

Figure 8 is a chromatogram of the 2-ME2 of the present invention produced in Example 2. The HPLC was run with an overloaded amount of sample, 270 µg (50 µl at 5.4 µl/ml).

Figure 9 is an expanded view of the chromatogram in Figure 8.

Figure 10 is a chromatogram of the pooled fractions from Example 2, assayed using a gradient (20 to 70% acetonitrile over 25 minutes, 1% acetic acid, and remainder water). 43.2 μg (8 μl of the 5.4 μl/ml sample) was injected.

Figure 11 is an expanded view of the chromatogram in Figure 10.

Figure 12 depicts a synthetic reaction scheme for the production of the 2-methoxyestradiol of the present invention, using estradiol as a starting material and employing bromine, a crown ether, and a blocking group on the 3- and 17-position hydroxyl oxygen atoms of the estradiol.

Figure 13 depicts a synthetic reaction scheme for the production of the 2-methoxyestradiol of the present invention, using estradiol as a starting material and employing bromination at the 2-position of the A ring of unblocked estradiol and a crown ether.

Figure 14 depicts a synthetic reaction scheme for the production of the 2-methoxyestradiol of the present invention, using estradiol as a starting material and employing a blocking group on the 3- and 17-position hydroxyl oxygen atoms of estradiol, nitration, and a Sandmeyer reaction.

Figure 15 depicts a synthetic reaction scheme for the production of the 2-methoxyestradiol of the present invention, using estrone as a starting material and employing a blocking group on the 3-position hydroxyl oxygen atom, nitration, and a Sandmeyer reaction.

Figure 16 depicts a synthetic reaction scheme for the production of the 2-methoxyestradiol of the present invention, using estradiol as a starting material and employing bromination at the 2-position of the A ring of unblocked estradiol and reaction with methanol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to 2-methoxyestradiol having a purity of greater than 98.0%, more preferably greater than 99.0%, and most preferably of 99.5% or higher. 2-ME2 can be obtained through synthesis methods or purification methods described herein that yield highly pure 2-ME2. The synthesis methods described herein may also be supplemented with the purification methods described herein to yield 2-ME2 having even greater purity.

Although the terms "2-methoxyestradiol" and 2-ME2 are specifically used herein, it should be understood that the methods disclosed herein can be used for synthesis or purification of other compounds, such as, but not limited to, estradiol and other structurally related steroids.

Methods of Synthesis

The present invention provides methods of synthesizing 2-ME2 to a purity of greater than 98.0%, more preferably greater than 99.0%, and most preferably of 99.5% or higher. The synthetic methods described herein can also be used, with minor modifications, to synthesize other 2- and 4- derivatives or analogues of estradiol, such as, for example, 4-methoxyestradiol and 4-hydroxyestradiol.

There are several synthetic approaches that can be taken to prepare 2-ME2 having a purity greater than 98.0%. Alternatively, 2-ME2 can be purified according to the following purification methods to have a purity greater than 98.0%. These different synthetic approaches utilize different starting materials and intermediates; consequently, different yields, side reactions and impurities will be obtained.

Two similar approaches employ estradiol as a starting material and utilize a brominated intermediate, as taught by Rao, P.N. *et al.*, Synthesis, 1977, 168 and Chen, S-H *et al.*, Steroids, 1986, 47, 63. The first approach is illustrated in Figure 12. The free hydroxyl groups of estradiol are protected with a blocking group. A wide range of blocking groups can be used in the present invention. These blocking groups include, but are not limited to, alkyl, aryl, aralkyl groups, and alkyl, aryl, and aralkyl group containing one or more heteroatoms. For example, protection can be accomplished using an alkyl halide, such as benzyl bromide, to form an alkyl ether. Appropriate conditions for hydroxyl protection include reaction of the estradiol and alkyl halide in the presence of NaH and TBAI, optionally in the presence of a solvent, such as dimethyl formamide (DMF). The protected estradiol is then reacted with bromine, for example, in the presence of acetic acid. Protection of the free hydroxyls during bromination gives a higher yield of the 2-brominated intermediate (about 70% vs. about 20% without the protecting groups) (*see* Cushman, M. *et al.*, J. Med. Chem. 1997, 40, 2323).

The bromine is then replaced with a methoxide group using a copper catalyst. For example, the brominated intermediate can be reacted with NaOMe in the presence of a copper catalyst, such as

CuI. The reaction is preferably conducted in a solvent, such as DMF, optionally in the presence of a promoter. Acceptable promoters, include, but are not limited to, crown ethers, such as benzo-15-crown-5.

Removal of the protecting groups, for example, by catalytic hydrogenation of the alkyl moiety, yields 2-ME2. Unfortunately, this synthetic route yields about 1-2% impurity of estradiol from the methoxylation step (a hydride quenches the reactive copper complex rather than a methoxide). The estradiol can be removed to undetectable levels by chromatography, such as described below, or significantly reduced by successive crystallization in chloroform.

Another synthetic method utilizing a brominated intermediate and employing estradiol as the starting material is illustrated by Figure 13. In this synthetic reaction, the estradiol is ring brominated without first blocking the hydroxyl groups. The bromine is then replaced with a methoxide using a copper catalyst in a manner similar to that described above.

In another approach, estradiol or estrone can be used as a starting material in a reaction scheme that utilizes nitro/amine intermediates (*see* Cushman, M. *et al.*, J. Med. Chem. 1995, 38, 2041). These synthetic approaches are illustrated in Figure 14 (estradiol starting material) and Figure 15 (estrone starting material). In these approaches, the free hydroxyl groups are protected. This protection can be accomplished, for example, using an alkyl halide, such as benzyl bromide, to form an alkyl ether. Appropriate conditions for hydroxyl protection include reaction of the starting material and alkyl halide in

the presence of NaH and TBAI, optionally in the presence of a solvent, such as dimethyl formamide (DMF).

The protected starting material is then nitrated, for example, with nitric acid and acetic acid or with nitric acid and sulfuric acid, to form the corresponding 2-nitro product. The nitro group is then reduced. Selective reduction can be accomplished by catalytic hydrogenation, for example, hydrogenation in the presence of Pd/C to produce the corresponding 2-amine. The catalytic reduction is optimally carried out for a period of one hour. Using Sandmeyer conditions (nitrous acid and sodium methoxide), the 2-amino group can be converted to the 2-methoxy substituent. Catalytic hydrogenation removes the protecting groups to give 2-ME2 when the starting material is estradiol and 2-methoxyestrone when the starting material is estrone. Reduction of the 17-keto group of 2-methoxyestrone with sodium borohydride yields 2-ME2.

Yet another method employs estradiol as the starting material and utilizes brominated intermediates. In this synthetic reaction, the estradiol is ring brominated without first blocking the hydroxyl groups. Bromination is accomplished, for example, with bromine and acetic acid in a solvent, such as THF. This reaction results in bromination at different sites on the ring, including multi-brominated species. The 2-bromo-estradiol can then be isolated from the other brominated intermediates, for example, by chromotography or crystallization, followed by replacement of the bromine with a methoxide. The bromine can be replaced with a methoxide group, for example, using sodium methoxide and methanol in the presence of a copper catalyst, such as CuI, in a manner similar to that described

above. Alternatively, the intermediates can be reacted to form the corresponding methoxides, followed by isolation of the 2-methoxyestradiol by the methods described above.

Methods of Purification

The present invention provides methods of purifying 2-ME2 to a purity of greater than 98.0%, more preferably greater than 99.0% and most preferably of 99.5% or higher. The 2-ME2 preparations preferably contain less than 0.03% estradiol, 0.02% or less 2-hydroxyestradiol, 0.02% or less 4-hydroxyestradiol, 0.02% or less 4-methoxyestradiol, and less than 0.02% estrone. Most preferably, the 2-ME2 preparations contain 0.01% or less estradiol, 0.02% or less 2-hydroxyestradiol, 0.01% or less 4-hydroxyestradiol, 0.01% or less 4-methoxyestradiol, and 0.01% or less estrone.

The purification methods of the present invention involve liquid chromatography on an adsorption/partition medium such as silica, using a solvent system comprising a polar and a non-polar solvent. The purification methods described herein can also be used, with minor modifications, to purify compounds similar to 2-ME2, such as, for example, 4-methoxyestradiol, 4-hydroxyestradiol, 2-hydroxyestradiol, estradiol, estrone, 2-methoxyestrone, and 4-methoxyestrone.

The Sample

The sample to be purified can be synthesized, or obtained from a biological source. The sample may be a commercially available 2-ME2 preparation, such as those sold by Sigma-Aldrich Chemicals of St. Louis, Missouri, Research Plus, Inc. of Bayonne, NJ, or Calbiochem

of San Diego, CA. The sample is preferably at least about 50% pure, more preferably about 75% pure, even more preferably about 90% pure, and most preferably about 98% pure. The sample can be subjected to other purification steps prior to the methods described herein, such as selective crystallization.

The sample is preferably dissolved into or solvent-exchanged into a loading solvent, as further described below. The sample is preferably at a concentration in the range of about 0.01 to 2 g/ml, preferably about 0.01 to 1 g/ml, more preferably about 0.05 to 0.2 g/ml.

Chromatographic Media

Silica is preferably used as the chromatographic medium. Silica gel of about 70-400 mesh is preferred, most preferably about 70-230 mesh, such as supplied by Merck and other vendors. The medium can be used loose, in batch chromatography, or packed into a column. Pre-packed columns, such as those sold by Biotage of Charlottesville, VA, can also be used. The medium should be equilibrated in an appropriate solvent before application of the sample to the medium, as further discussed below.

Column Dimensions

The chromatographic methods described herein can be achieved using batch or column chromatography. In batch chromatography, the sample and the chromatographic medium are combined in a container for a period of time sufficient to allow the 2-ME2 to be retained by the medium. The medium is then preferably washed with wash solvent. Elution solvents are then applied to the

medium. After the loading, wash, and elution steps, the solvent is removed from the medium, such as by filtration.

For column chromatography, a column having appropriate dimensions is packed with the chromatography medium. The column, after equilibration with appropriate solvent, is loaded with sample by applying the sample to the top, or entrance, of the column. The ratio of the sample volume to column diameter should preferably be between about 0.2 to 3 ml/cm, and more preferably between about 0.5 and 1.5 ml/cm for best results.

Solvents

A solvent system including a polar solvent, such as methanol (MeOH), and a non-polar solvent, such as chloroform ($CHCl_3$), is used. Other polar solvents that can be used include, but are not limited to, tetrahydrofuran (THF), ethyl acetate, isopropanol, ethanol, propanol, and combinations thereof. Other non-polar solvents that can be used include, but are not limited to, hexane, dichloromethane, cyclohexane, pentane, and combinations thereof. More specifically, solvent systems that can be used include THF/hexane, ethyl acetate/hexane, isopropanol/hexane, ethanol/$CHCl_3$, propanol/$CHCl_3$, isopropanol/$CHCl_3$, and combinations thereof.

The sample is soluble in the polar solvent. Some amount of the polar solvent, generally about 10%, is needed to render the sample soluble in the loading solvent. The loading solvent thus will include up to about 10% polar solvent and about 90% non-polar solvent.

After the sample is loaded onto the medium, the medium may be washed with a wash solvent that will wash contaminants off the medium but will not elute the 2-ME2. The wash solvent comprises mostly non-polar solvent, with enough polar solvent to prevent the 2-ME2 from precipitating but not enough polar solvent to elute the 2-ME2.

The sample is eluted with elution solvent that contains enough polar solvent to elute the 2-ME2 from the medium. The elution solvent may be applied as a step gradient or as a linear gradient, as described below.

Column Conditions

The wash and elution solvents can be applied to the column in a step gradient or in a linear gradient. In a preferred embodiment, the solvents are applied using a step gradient of increasing concentration of polar solvent.

The column can be operated using the force of gravity or can be operated with a pump that forces liquids through the column. The rate at which the column is operated will depend upon the volume and dimensions of the column and the silica gel particle size. In general, the column can be operated at a rate from about 0.5 to 5 ml/min.

The eluant can be monitored visually or, monitored with a spectrophotometer at a wavelength of about 288 nm, which is the absorbance maximum of 2-ME2, and collected as the 2-ME2 elutes from the column.

The column can optionally be operated under pressure and can optionally be heated. Preparative high performance liquid chromatography (HPLC), either normal phase or reversed phase, or fast performance liquid chromatography (FPLC) techniques can be used. Commercial preparative chromatography apparatus, such as that sold by Biotage of Charlottesville, VA, can also be used. Other known methods of improving column efficiency and/or speed can also be employed.

Sample Collection and Treatment

The eluant can be collected as fractions which are then assayed for 2-ME2 content and purity. These fractions can then be combined to achieve the desired purity of 2-ME2. The fractions can be assayed for purity using reverse phase HPLC with a C-18 column (Waters) and an isocratic solvent system of 30:69:1 acetonitrile:water:acetic acid. Other systems can be used for sample analysis, such those that use solvent gradients instead of the isocratic solvent system; those that use trifluoroacetic acid or formic acid rather than acetic acid; and those that use methanol rather than acetonitrile.

Alternatively, or in combination, the eluant can be monitored in real time and sample collection begun when 2-ME2 of desired purity elutes from the column.

The solvent is removed from the pooled fractions by use of a vacuum and/or other solvent removal methods. Lyophilization and other evaporative methods can be used.

Preferred Embodiment

In a preferred embodiment the medium is silica, which is packed into a column. The sample is dissolved in a mixture of $CHCl_3$ and MeOH, with enough MeOH to solubilize the 2-ME2, generally about 90:10 $CHCl_3$:MeOH. The elution conditions are a step gradient from 99:1 $CHCl_3$:MeOH to 98:2 $CHCl_3$:MeOH.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLE 1

Commercially available samples of 2-ME2 were assayed by analytical HPLC to determine their overall purity and the amounts of certain contaminants, namely estradiol, 4-hydroxyestradiol, 4-methoxyestradiol, 2-hydroxyestradiol, estrone, and 2-methoxyestrone.

These analytical HPLC chromatograms were generated using reverse phase HPLC with a C-18 column (Waters) and a solvent gradient (20 to 50% acetonitrile over 30 minutes, 50 to 80% acetonitrile over 5 minutes, 1% acetic acid, remainder water). The eluant was monitored at a wavelength of 288 nm. In this system 2-ME2 elutes at about 21.5 minutes, estradiol elutes at about 20.0 minutes, estrone

elutes at about 23.2 minutes, 4-hydroxyestradiol elutes at about 15.0 minutes, 4-methoxyestradiol elutes at about 20.4 minutes, 2-hydroxyestradiol elutes at about 15.4 minutes, and 2-methoxyestrone elutes at about 24.4 minutes.

The chromatogram of a sample from Sigma-Aldrich Chemicals of St. Louis, Missouri is shown in Figure 1. The sample has an overall purity of 99.2% but has contaminating estradiol of about 0.034%, an unacceptable amount. Figure 2 is an expanded view of the chromatogram of Figure 1.

Figure 3 is a chromatogram of a sample obtained from Research Plus, Inc. of Bayonne, NJ that shows that the 2-ME2 has a purity of 98.6%. The automatic peak calculator and the expanded view shown in Figure 4 show that the preparation contains 0.024% estrone, an unacceptable amount of this contaminant. Other samples tested showed 2-ME2 purity less than 98%, including a second batch obtained from Research Plus (97.2% 2-ME2) and a sample from CalBiochem of San Diego, California (91.8% 2-ME2).

Table 1, below, illustrates the purity and contaminants of these commercially available samples of 2-ME2 and the purified 2-ME2 of the present invention.

TABLE 1

	Sigma	Research Plus, Lot #1	Research Plus, Lot #2	Calbiochem	PharmEco	purified
2-ME2	99.18	98.61	97.17	91.80	97.80	99.98
estradiol	0.03	n.d.	n.d.	1.78	2.2	less than 0.01%
estrone	n.d.	0.02	0.43	0.011		n.d.
4-hydroxy-estradiol	n.d.	n.d.	n.d.	n.d.		n.d.
4-methoxy-estradiol	0.49	0.121	0.18	1.99		n.d.
2-hydroxy-estradiol	n.d.	n.d.	n.d.	0.06		n.d.
2-methoxy-estrone	n.d.	n.d.	n.d.	0.20		n.d.

* n.d. means none was detected.

EXAMPLE 2

A 55 cm diameter (60 cm height) glass column was packed with 600 g silica gel (70-230 mesh from Merck) in 90:10 $CHCl_3$:MeOH. The column was washed with one liter of $CHCl_3$ to remove the MeOH from the column.

The sample was 3.5 g 2-ME2 in 60 ml 90:10 $CHCl_3$:MeOH. The 2-ME2 was obtained from PharmEco Laboratories, Inc. of Lexington, MA, and was 97.8% pure as determined by analytical HPLC (Figure 5). The peak eluting at 10.917 is estradiol (2.2%).

Analytical HPLC of the starting material, the column fractions, and the pooled product was performed using reverse phase HPLC with a C-18 column (Waters) and an isocratic gradient of 30:69:1

acetonitrile:water:acetic acid, which provides good separation of 2-ME2 and estradiol. The eluant was monitored at a wavelength of 288 nm.

The sample was applied to the top of the column and allowed to enter the bed volume. The column was eluted with one liter of 99:1 $CHCl_3$:MeOH and then 1.5 L of 98:2 $CHCl_3$:MeOH. Fractions of 50 ml each were collected and 15 fractions containing 2-ME2 were assayed for 2-ME2 purity using the analytical isocratic HPLC system described above. Nine to ten fractions that showed no amount of estradiol were pooled together and solvent was evaporated. After drying under vacuum for 4 hours, 3.2 g of yellow/white crystals were collected, for a 91% yield.

Purity of the pooled fractions was determined by analytical HPLC to be 99.984%, using the isocratic technique described above. The HPLC chromatograms are shown in Figures 6 through 9. Figure 6 was generated with a non-overloaded amount of sample, 75.6 µg (14 µl at 5.4 µl/ml). Figure 7 is an expanded view of the chromatogram of Figure 6. The automatic peak finder calculated the 2-ME2 to be 100.0%, although a small, unknown impurity peak is seen in the expanded view, eluted prior to the 2-ME2. Figure 8 was generated with an overloaded amount of sample, 270 µg (50 µl at 5.4 µl/ml). Figure 9 is an expanded view of the chromatogram of Figure 8. The automatic peak finder calculated the 2-ME2 to be 99.984% pure, with a small, unknown, impurity that eluted prior to the 2-ME2, and after estradiol, that was calculated to be 0.016%. The expanded view shown in Figure 9 shows this impurity peak more clearly and shows that the 2-ME2 peak is very clean.

The pool was also assayed using a gradient (20 to 70% acetonitrile over 25 minutes, 1% acetic acid, and remainder water). 43.2 μg (8 μl of the 5.4 μl/ml sample) was injected. The chromatogram is shown in Figure 10. The automatic peak finder calculated the 2-ME2 to have a purity of 99.825%. However, when an artifact peak, present in a blank run, at 29.45 minutes is removed from consideration, the calculated purity is 99.9%. Figure 11 is an expanded view of this chromatogram. The unknown impurity at 13.43 minutes was calculated to be 0.012%. If estradiol were present, it would elute between the unknown purity and the 2-ME2 peak. If estradiol is present, therefore, it can be present at no more than 1/3 to 1/4 of the amount of the unknown peak. Accordingly, the estradiol amount was estimated to be no more than 0.004%. The preparation contained 0.02% or less 2-hydroxy-estradiol, 0.01% or less 4-hydroxy-estradiol, 0.01% or less 4-methoxy-estradiol, and 0.01% or less estrone, as demonstrated by the lack of any measurable peaks at the expected retention times.

The purified sample was also subjected to elemental analysis and the results are shown in Table 2.

TABLE 2: Elemental Analysis

Element	Theoretical	Found
Carbon	75.46	75.21
Hydrogen	8.67	8.65
Oxygen	15.87	16.13 (obtained by difference)
Chlorine	0.00	0.0

The above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patents, patent applications and publications, are incorporated herein by reference.

What is claimed is:

1. A composition comprising 2-methoxyestradiol having a purity greater than 99.5%.

2. The composition of Claim 1, containing less than 0.03% estradiol and less than 0.02% estrone.

3. The composition of claim 2, containing less than 0.01% estradiol and less than 0.01% estrone.

4. The composition of Claim 2, further containing less than 0.02% 2-hydroxyestradiol.

5. The composition of Claim 2, further containing less than 0.02% 4-hydroxyestradiol.

6. The composition of Claim 2, further containing less than 0.02% 4-methoxyestradiol.

7. The composition of Claim 1, containing 0.01% or less estradiol, 0.02% or less 2-hydroxyestradiol, 0.01% or less 4-hydroxyestradiol, 0.01% or less 4-methoxyestradiol, and 0.01% or less estrone.

8. A composition comprising 2-methoxyestradiol having a purity greater than 98.0% and containing less than 0.03% estradiol and less than 0.02% estrone.

9. The composition of Claim 8, containing less than 0.01% estradiol and less than 0.01% estrone.

10. The composition of Claim 8, containing 0.01% or less estradiol, 0.02% or less 2-hydroxyestradiol, 0.01% or less 4-hydroxyestradiol, 0.01% or less 4-methoxyestradiol, and 0.01% or less estrone.

11. The composition of Claim 8, wherein the 2-methoxyestradiol has a purity greater than 99.0%.

12. The composition of Claim 11, containing less than 0.01% estradiol and less than 0.01% estrone.

13. The composition of Claim 11, containing 0.01% or less estradiol, 0.02% or less 2-hydroxyestradiol, 0.01% or less 4-hydroxyestradiol, 0.01% or less 4-methoxyestradiol, and 0.01% or less estrone.

14. A method for purifying 2-methoxyestradiol to a produce a 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

adding a solution comprising 2-methoxyestradiol to a chromatography medium; and

eluting the 2-methoxyestradiol off of the medium with a solvent system comprising a polar solvent and a non-polar solvent.

15. The method of claim 14, wherein the medium is silica and wherein the 2-methoxyestradiol is eluted using a step gradient of 99:1 $CHCl_3$:MeOH to 98:2 $CHCl_3$:MeOH.

16. A method for producing 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

protecting the 3- and 17-hydroxyl groups of estradiol;

reacting the protected estradiol with bromine and acetic acid to produce a 2-brominated derivative of estradiol;

reacting the 2-brominated derivative of estradiol with sodium methoxide in the presence of a copper catalyst;

removing the protecting groups on the 3- and 17-hydroxyl groups to produce 2-methoxyestradiol; and

purifying the 2-methoxyestradiol using liquid chromatography on an adsorption/partition medium with a solvent system comprising a polar and a nonpolar solvent.

17. A method for producing 2- methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

ring-brominating estradiol by reacting estradiol with bromine in the presence of acetic acid to produce a ring-brominated intermediate;

reacting the ring-brominated intermediate with sodium methoxide in the present of a copper catalyst to produce 2-methoxyestradiol; and

purifying the 2-methoxyestradiol using liquid chromatography on an adsorption/partition medium with a solvent system comprising a polar and a nonpolar solvent.

18. A method for producing 2- methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

protecting the 3- and 17-hydroxyl groups of estradiol;

reacting the protected estradiol with nitric acid and acetic acid to produce a 2-nitro derivative of estradiol;

reducing the 2-nitro derivative of estradiol to produce the corresponding 2-amino derivative of estradiol;

reacting the 2-amino derivative of estradiol under Sandmeyer conditions to produce a 3-,17-hydroxyl protected 2-methoxyestradiol; and

removing the protecting groups on the 3- and 17-hydroxyl groups to produce 2-methoxyestradiol.

19. A method for producing 2- methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

protecting the 3-hydroxyl group of estrone;

reacting the protected estrone with nitric acid and acetic acid to produce a 2-nitro derivative of estrone;

reducing the 2-nitro derivative of estrone to produce the corresponding 2-amino derivative of estrone;

reacting the 2-amino derivative of estrone under Sandmeyer conditions to produce a 3-hydroxyl protected 2-methoxyestrone;

removing the protecting group on the 3-hydroxyl group to produce 2-methoxyestrone; and

reducing the 17-keto group of 2-methoxyestrone to produce 2-methoxyestradiol.

20. A method for producing 2- methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone comprising:

brominating estradiol in the presence of acetic acid to produce a mixture of ring-brominated estradiols;

isolating 2-bromoestradiol from the mixture of estradiols; and

reacting the 2-bromoestradiol with sodium methoxide in the presence of a copper catalyst to produce 2-methoxyestradiol.

21. 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone produced by the process comprising:

protecting the 3- and 17-hydroxyl groups of estradiol;

reacting the protected estradiol with bromine and acetic acid to produce a 2-brominated derivative of estradiol;

reacting the 2-brominated derivative of estradiol with sodium methoxide in the presence of a copper catalyst;

removing the protecting groups on the 3- and 17-hydroxyl groups to produce 2-methoxyestradiol; and

purifying the 2-methoxyestradiol using liquid chromatography on an adsorption/partition medium with a solvent system comprising a polar and a nonpolar solvent.

22. 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone produced by the process comprising:

ring-brominating estradiol by reacting estradiol with bromine in the presence of acetic acid to produce a ring-brominated intermediate;

reacting the ring-brominated intermediate with sodium methoxide in the present of a copper catalyst to produce 2-methoxyestradiol; and

purifying the 2-methoxyestradiol using liquid chromatography on an adsorption/partition medium with a solvent system comprising a polar and a nonpolar solvent.

23. 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone produced by the process comprising:

protecting the 3- and 17-hydroxyl groups of estradiol;

reacting the protected estradiol with nitric acid and acetic acid to produce a 2-nitro derivative of estradiol;

reducing the 2-nitro derivative of estradiol to produce the corresponding 2-amino derivative of estradiol;

reacting the 2-amino derivative of estradiol under Sandmeyer conditions to produce a 3-,17-hydroxyl protected 2-methoxyestradiol; and

removing the protecting groups on the 3- and 17-hydroxyl groups to produce 2-methoxyestradiol.

24. 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone produced by the process comprising:

protecting the 3-hydroxyl group of estrone;

reacting the protected estrone with nitric acid and acetic acid to produce a 2-nitro derivative of estrone;

reducing the 2-nitro derivative of estrone to produce the corresponding 2-amino derivative of estrone;

reacting the 2-amino derivative of estrone under Sandmeyer conditions to produce a 3-hydroxyl protected 2-methoxyestrone;

removing the protecting group on the 3-hydroxyl group to produce 2-methoxyestrone; and

reducing the 17-keto group of 2-methoxyestrone to produce 2-methoxyestradiol.

25. 2-methoxyestradiol having a purity greater than 98% and containing less than 0.03% estradiol and less than 0.02% estrone produced by the process comprising:

brominating estradiol in the presence of acetic acid to produce a mixture of ring-brominated estradiols;

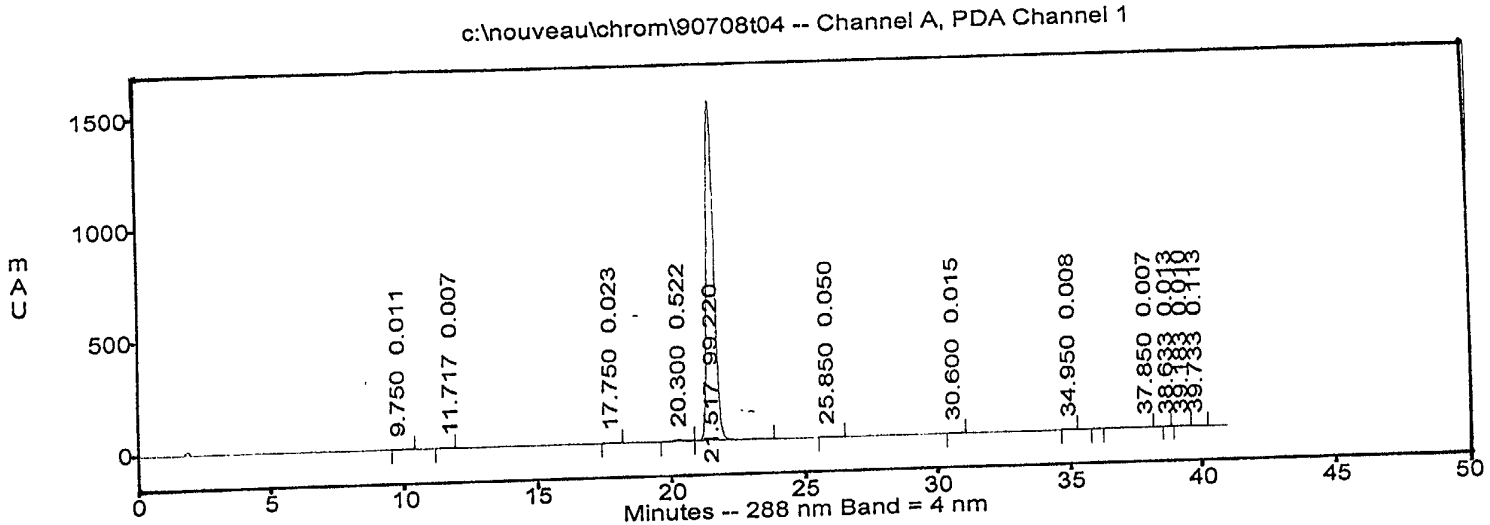
isolating 2-bromoestradiol from the mixture of estradiols; and

reacting the 2-bromoestradiol with sodium methoxide in the presence of a copper catalyst to produce 2-methoxyestradiol.

ABSTRACT OF THE DISCLOSURE

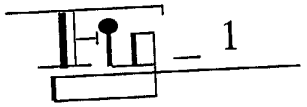
2-methoxyestradiol having greater than 98% purity is obtained by synthetic or purification methods. This highly pure 2-methoxy estradiol, lacking estrogenic components, is particularly suitable for clinical use in humans. The purification methods of the invention involve the use of liquid-solid chromatography (LSC) to separate 2-ME2 from other compounds. The chromatographic media is preferably silica. The solvent system comprises a non-polar solvent, such as chloroform, and a polar solvent, such as methanol.

Atty Docket: 05213-0541



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	9.75	3495	0.011
2	11.72	2337	0.007
3	17.75	7088	0.023
4	20.30	163323	0.522
5	21.52	31072058	99.220
6	25.85	15540	0.050
7	30.60	4828	0.015
8	34.95	2586	0.008
9	37.85	2242	0.007
10	38.63	4186	0.013
11	39.18	3270	0.010
12	39.73	35352	0.113
Totals :		31316304	100.000



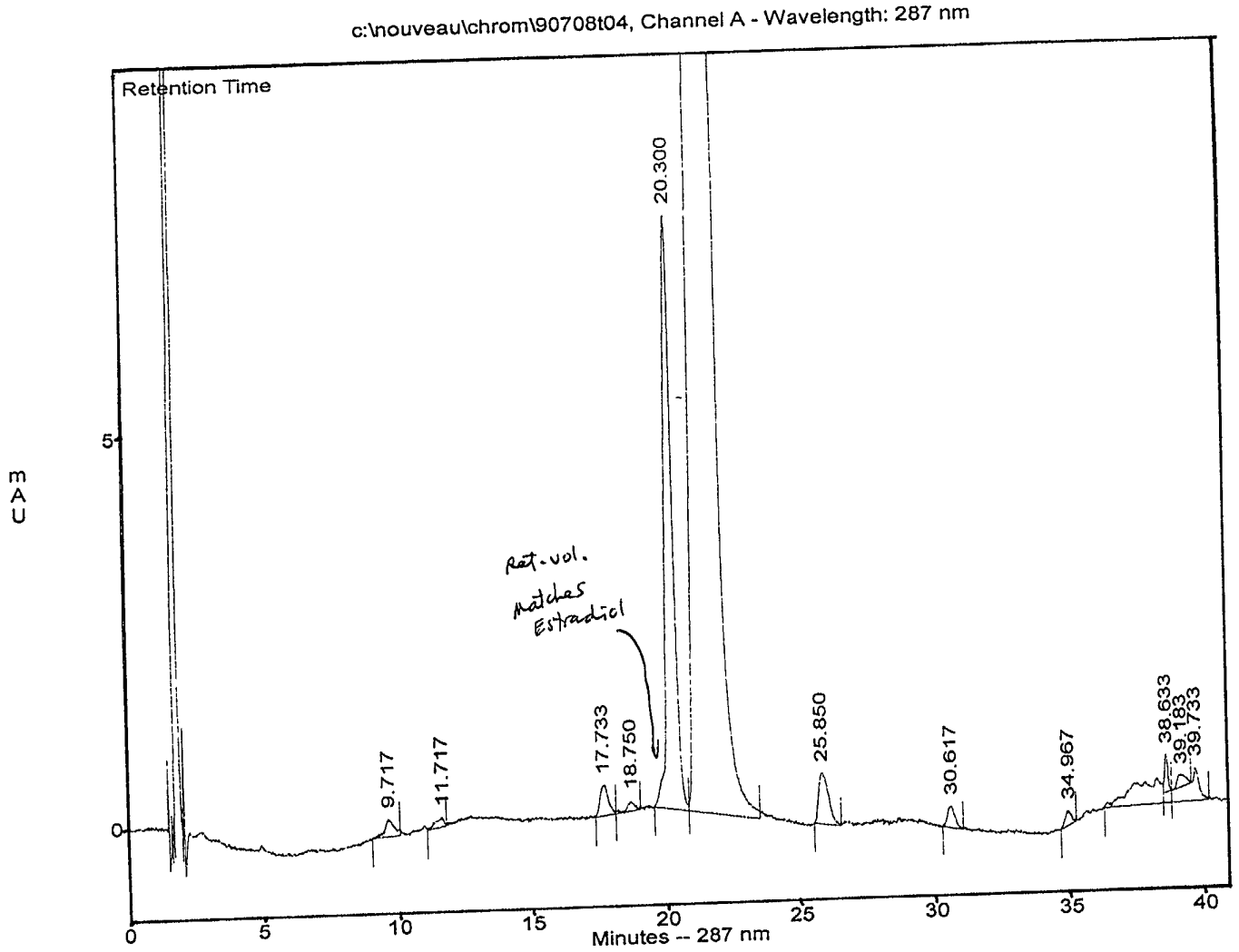
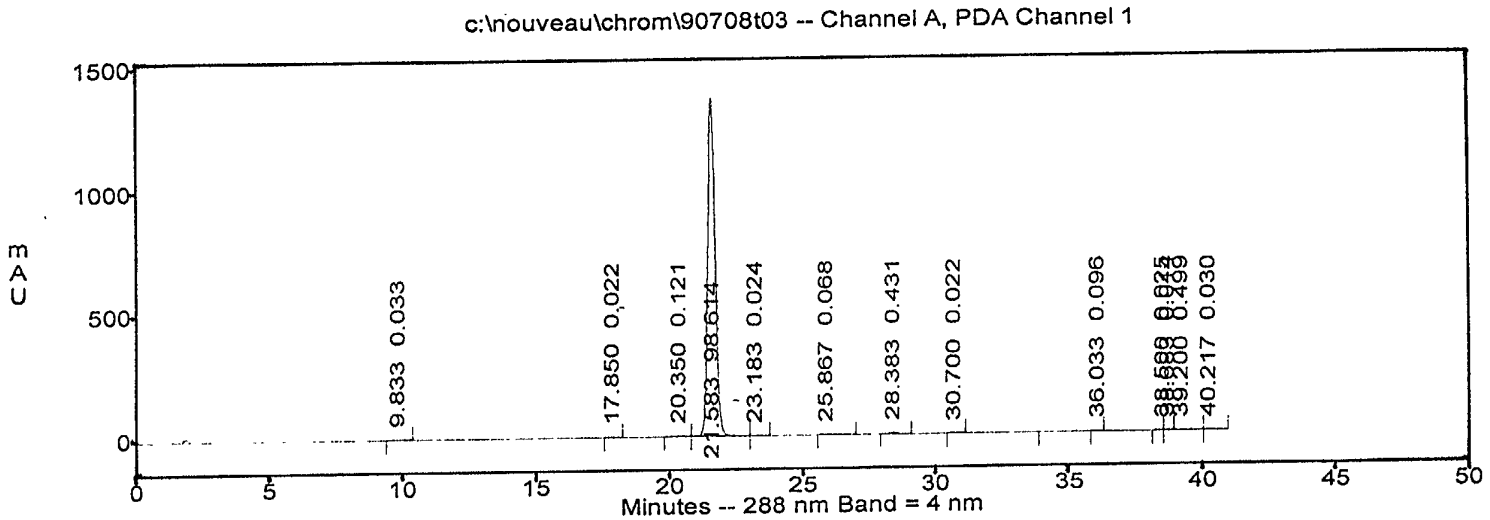


Fig. 2



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	9.83	8932	0.033
2	17.85	5885	0.022
3	20.35	32561	0.121
4	21.58	26490718	98.614
5	23.18	6510	0.024
6	25.87	18219	0.068
7	28.38	115757	0.431
8	30.70	6012	0.022
9	36.03	25812	0.096
10	38.50	6762	0.025
11	38.68	3699	0.014
12	39.20	134115	0.499
13	40.22	7956	0.030
Totals :		26862936	100.000

Fig 3

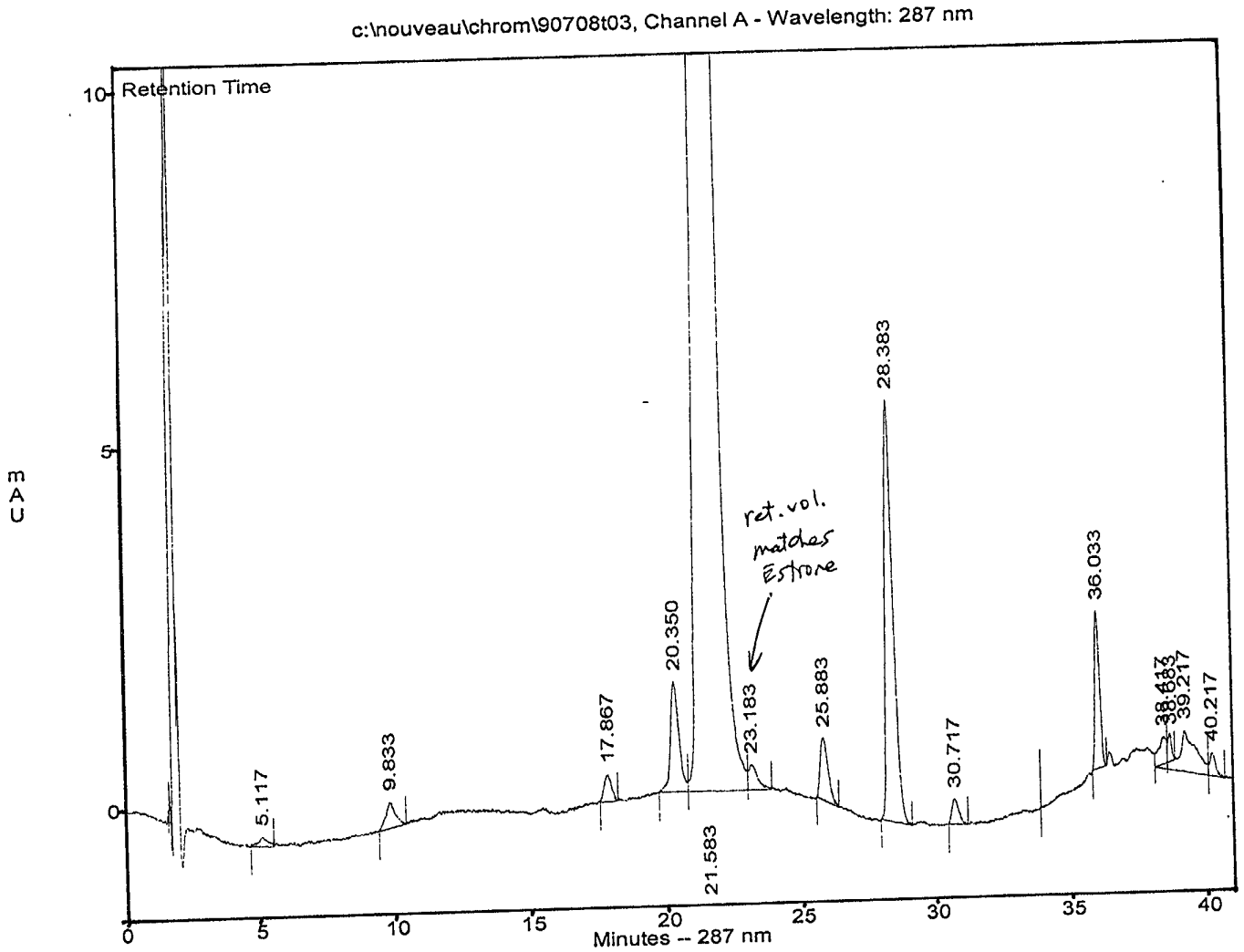
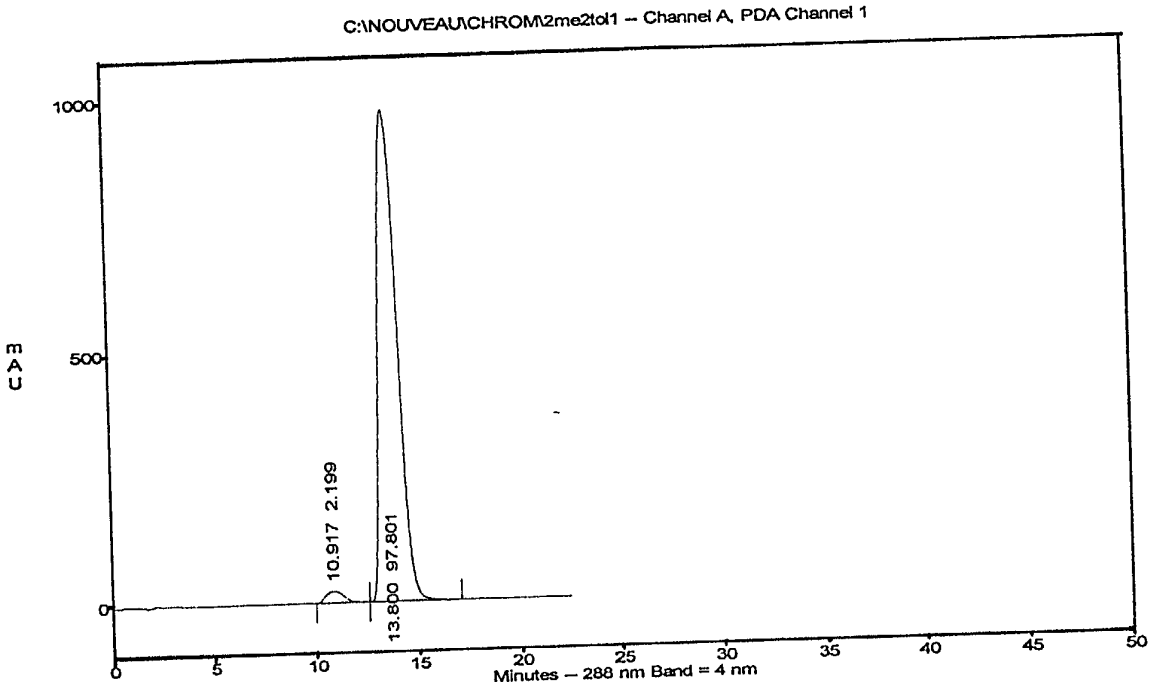


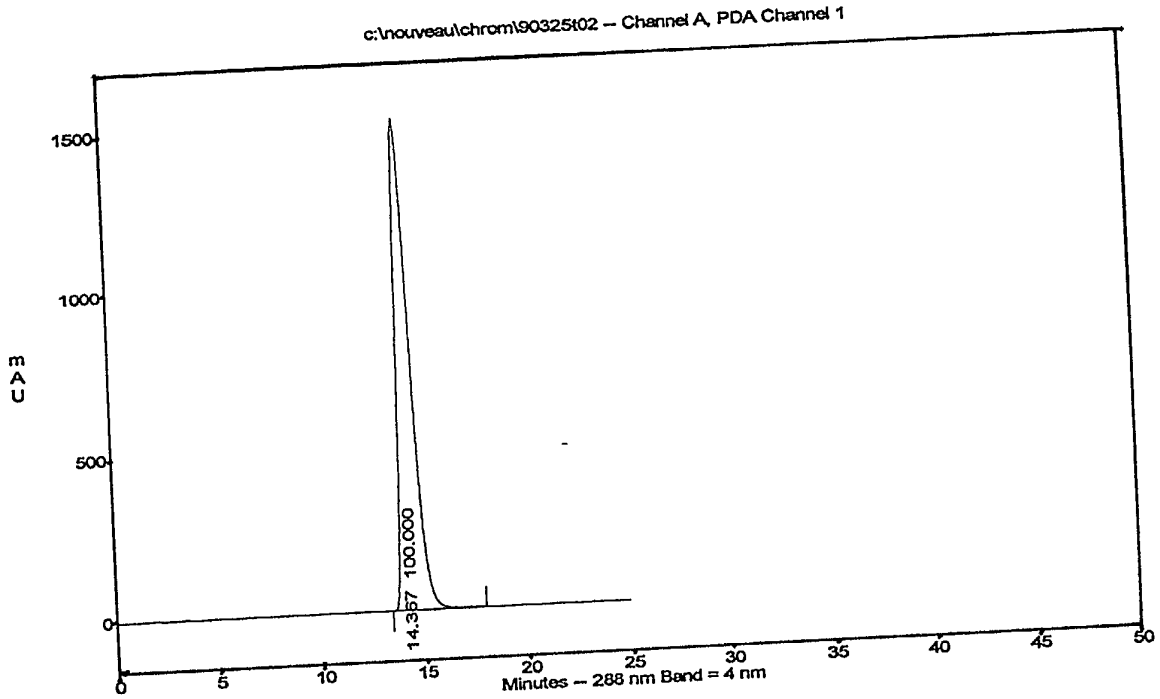
Fig_4



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	10.92	1393277	2.199
2	13.80	61954588	97.801
Totals :		63347864	100.000

Fig_5



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	14.37	71406720	100.000
Totals :		71406720	100.000

Fig_6

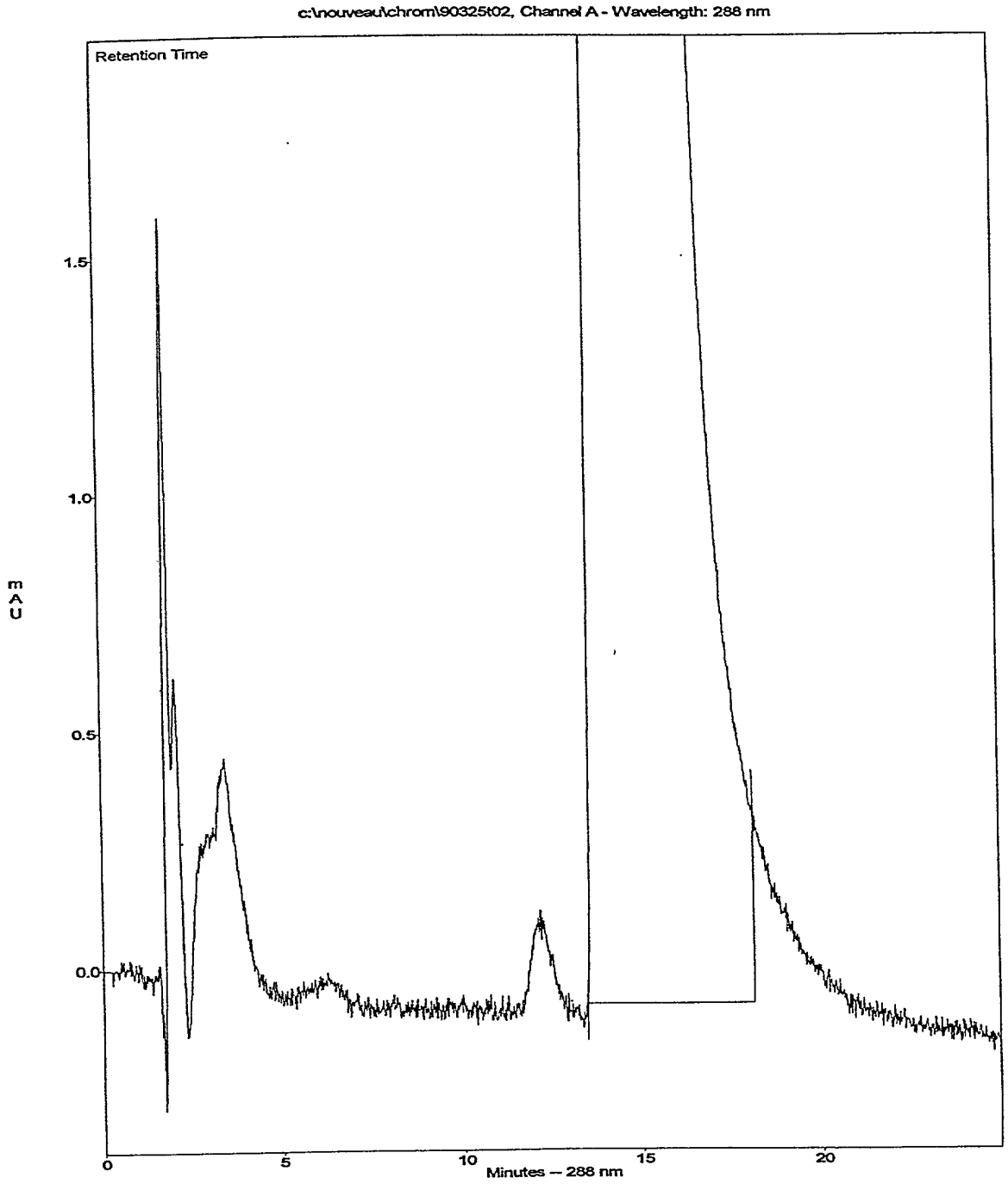
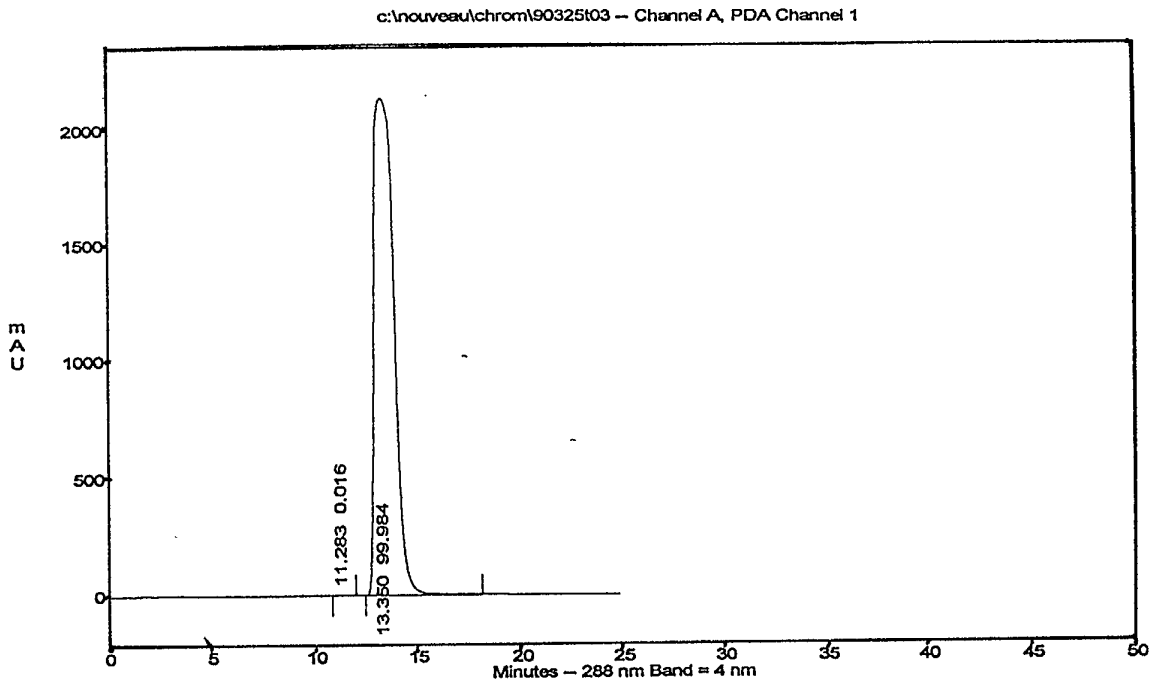

c:\nouveau\chrom\90325t02, Channel A - Wavelength: 288 nm
Retention Time
1.5
1.0
0.5
0.0
m
A
U
0
5
10
15
20
Minutes -- 288 nm

Fig_7



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	11.28	22624	0.016
2	13.35	142193616	99.984
Totals :		142216240	100.000

Fig_8

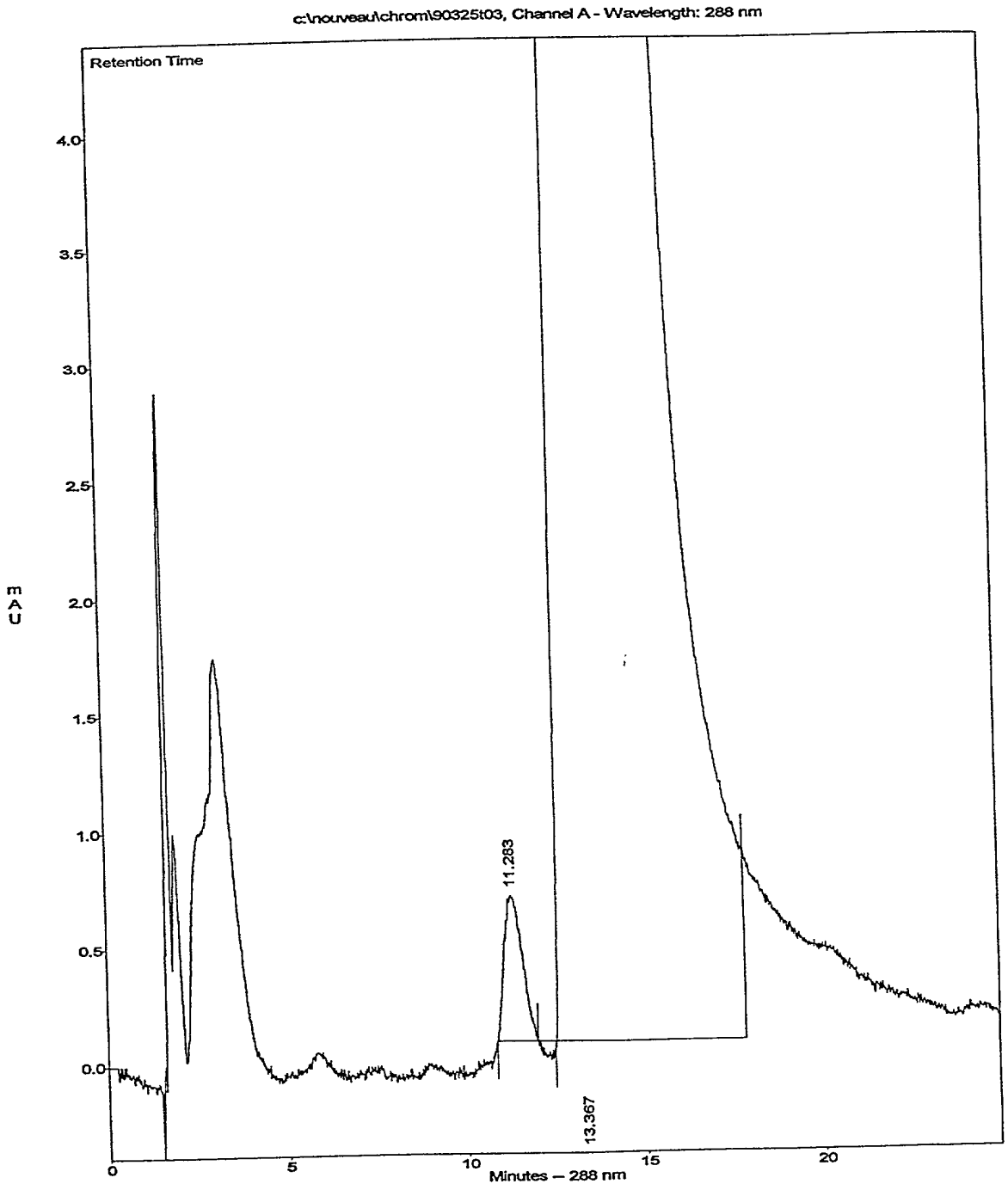
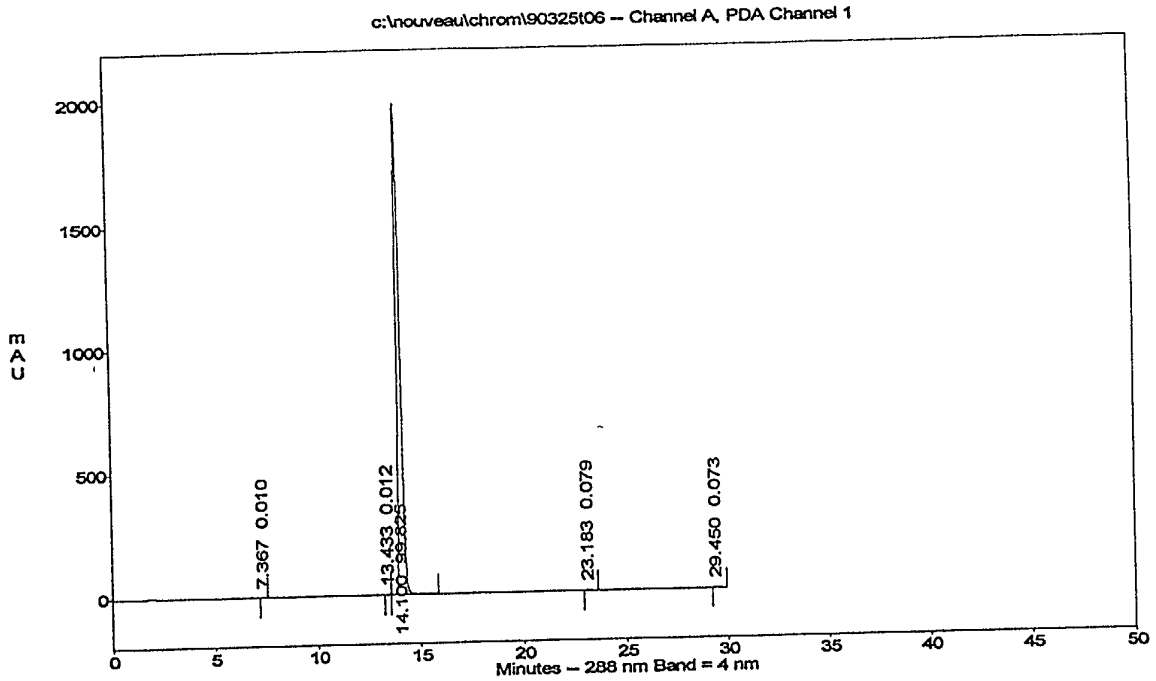

c:\nouveau\chrom\90325t03, Channel A - Wavelength: 288 nm
Retention Time
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
m
A
U
11.283
13.367
0
5
10
15
20
Minutes – 288 nm

Fig. 9



Channel A Results -- PDA Channel 1, 288 nm, 4 nm Band

Peak	Time	Area	Area %
1	7.37	2518	0.010
2	13.43	3186	0.012
3	14.10	25794770	99.825
4	23.18	20448	0.079
5	29.45	18953	0.073
Totals :		25839876	100.000

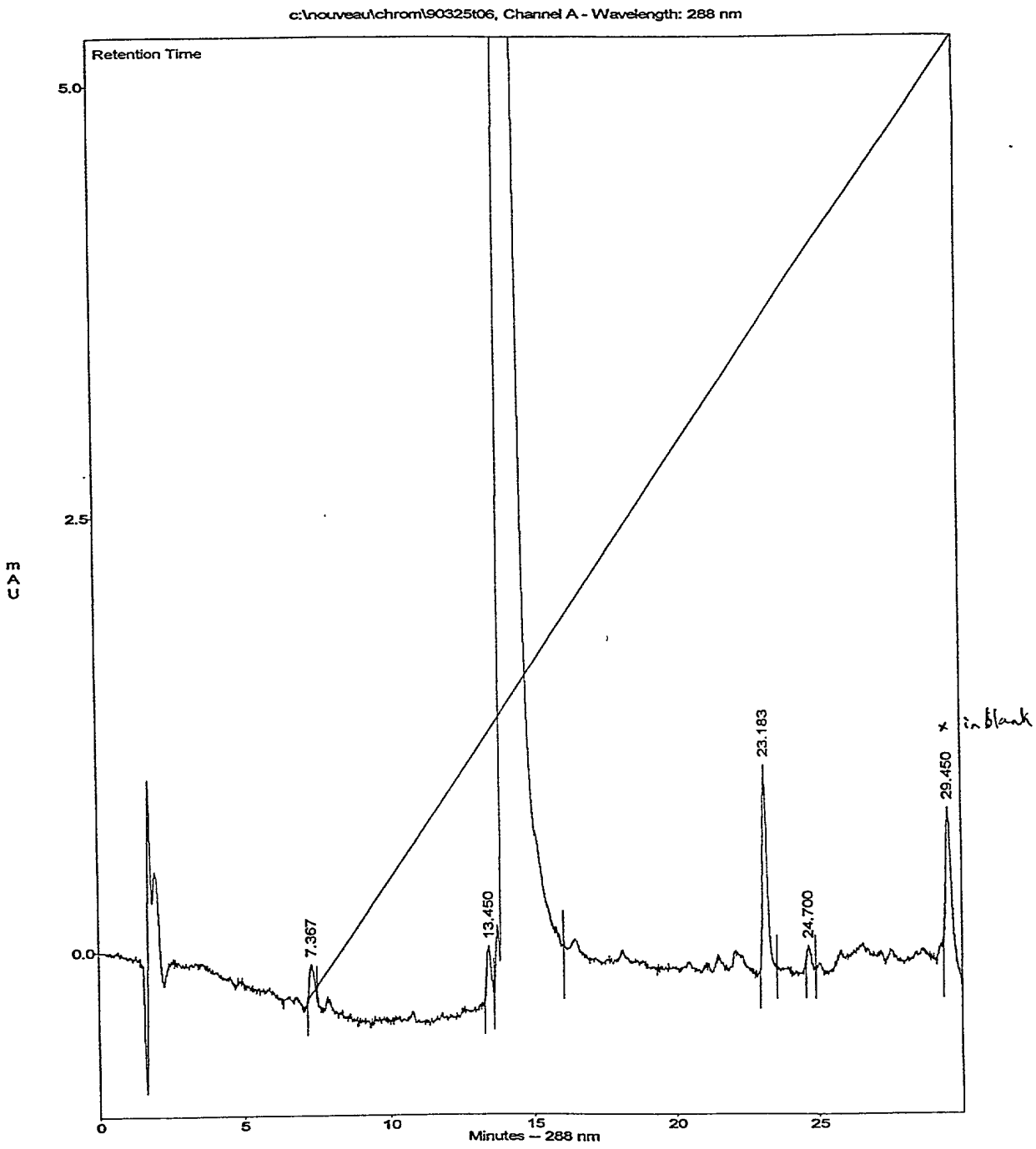

c:\nouveau\chrom\90325t06, Channel A - Wavelength: 288 nm
Retention Time
5.0
2.5
0.0
mAU
7.367
13.450
23.183
24.700
29.450
x : blank
0
5
10
15
20
25
Minutes – 288 nm

Fig. 11

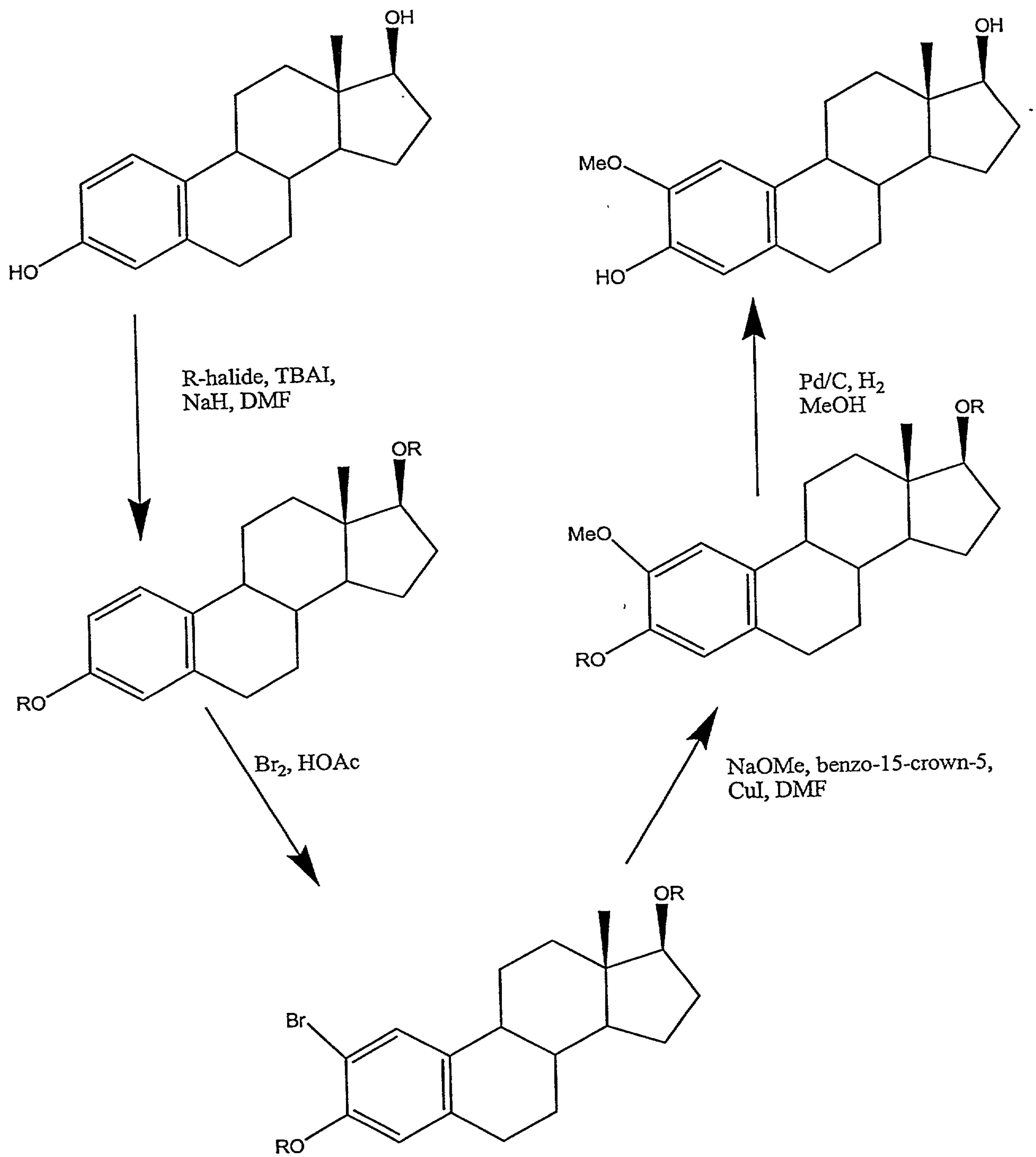

OH
HO
R-halide, TBAI,
NaH, DMF
OR
RO
Br$_2$, HOAc
Br
OR
RO
NaOMe, benzo-15-crown-5,
CuI, DMF
MeO
OR
RO
Pd/C, H$_2$
MeOH
OH
MeO
HO

Fig _12

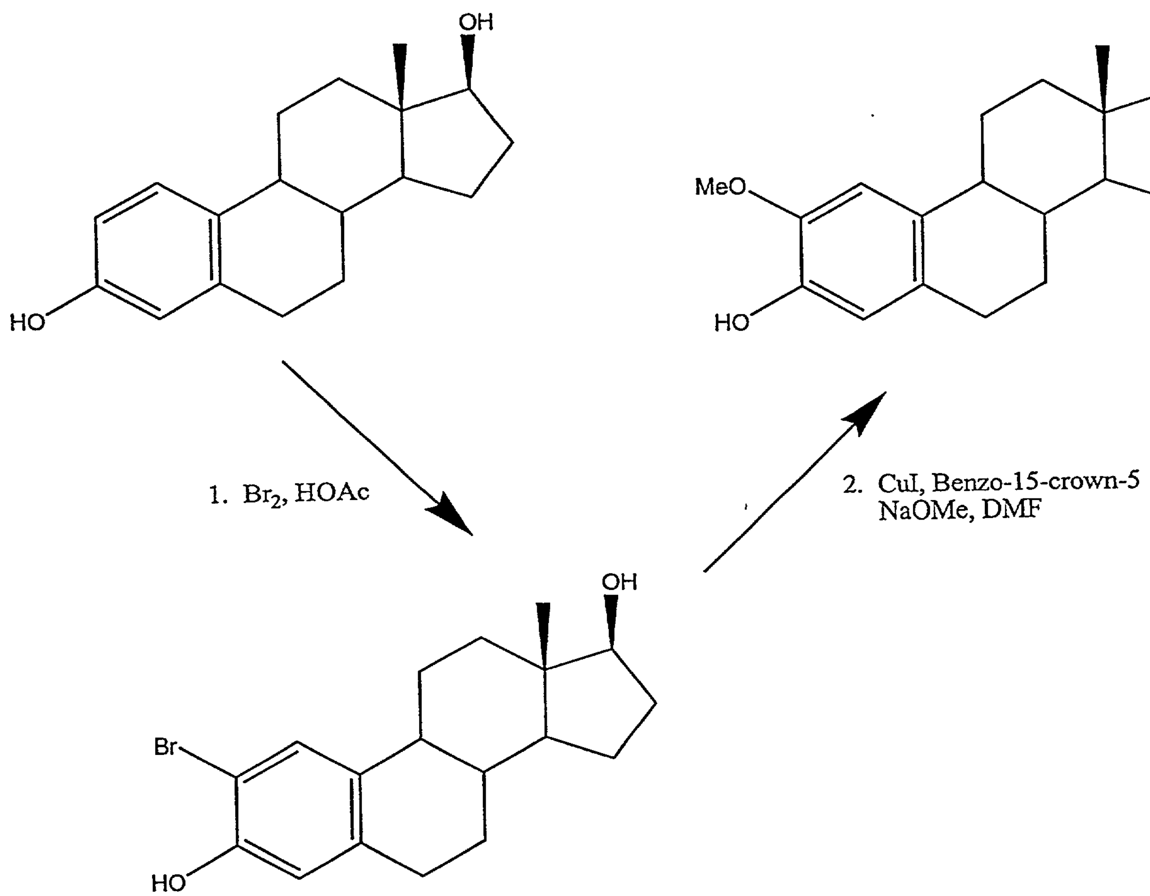
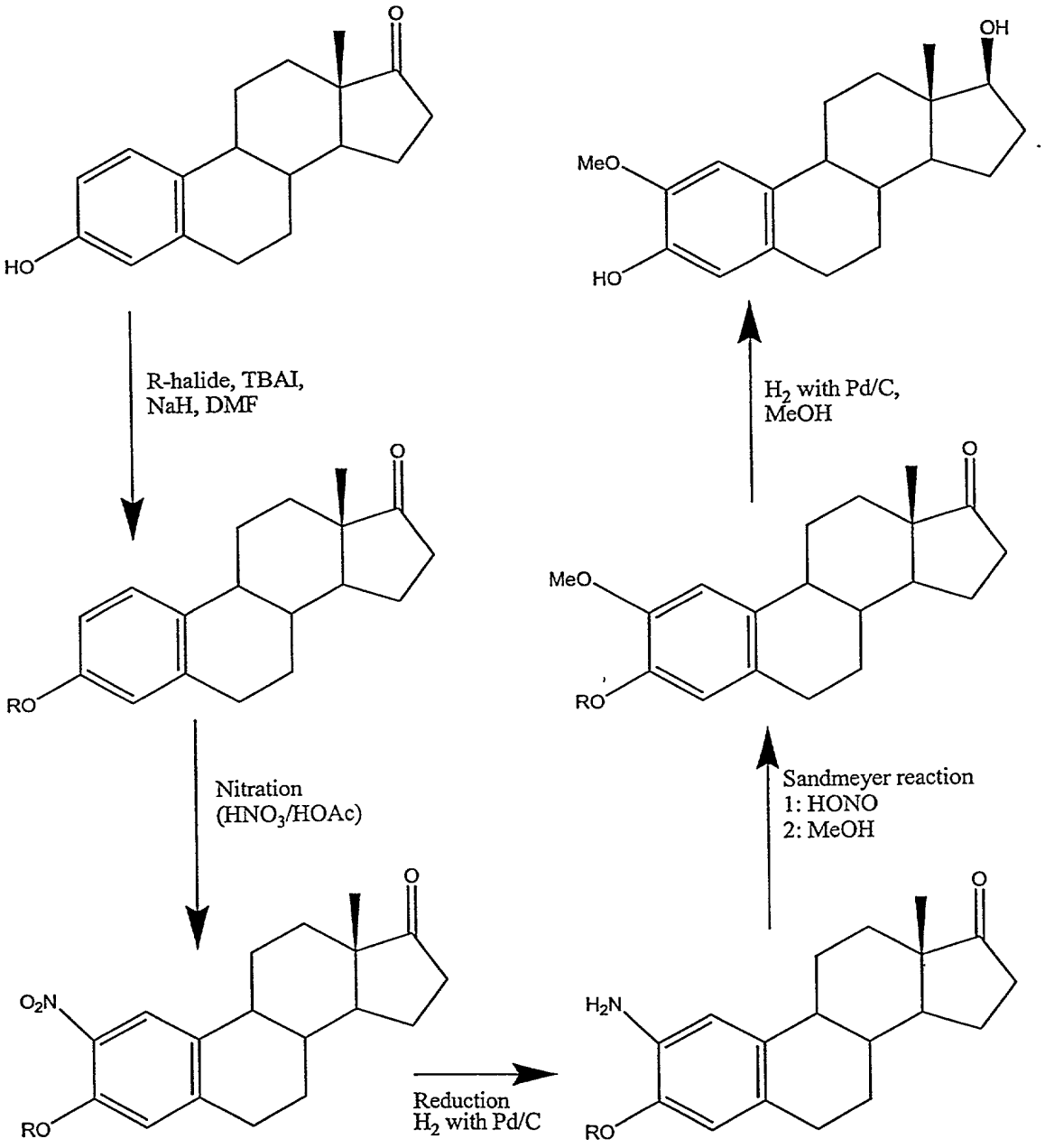

OH
HO
1. Br_2, HOAc
OH
Br
HO
2. CuI, Benzo-15-crown-5
NaOMe, DMF
OH
MeO
HO

Fig_13


HO
O
R-halide, TBAI,
NaH, DMF
RO
Nitration
(HNO3/HOAc)
O2N
RO
Reduction
H2 with Pd/C
H2N
RO
Sandmeyer reaction
1: HONO
2: MeOH
MeO
RO
H2 with Pd/C,
MeOH
MeO
HO
OH

14

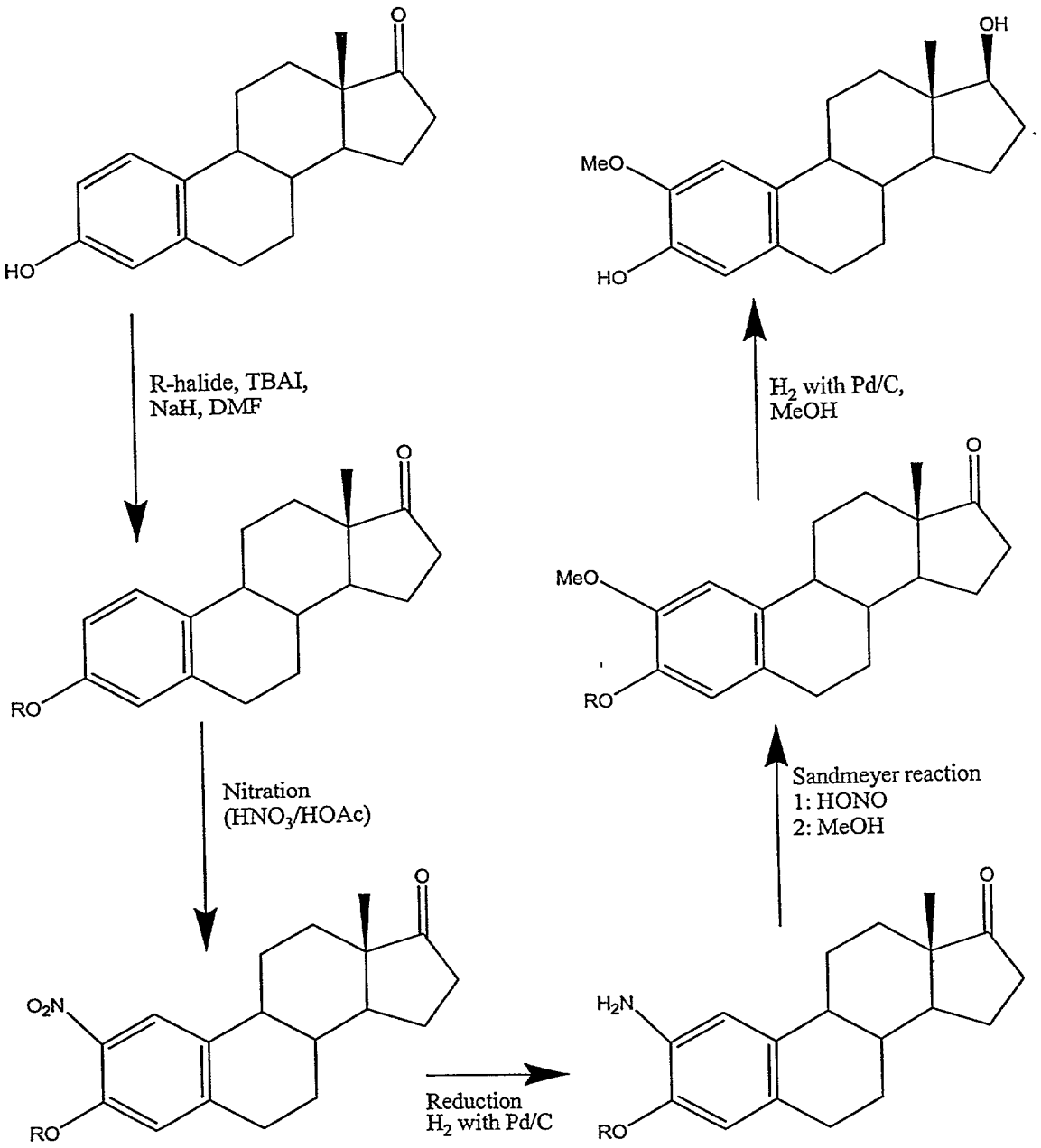

O
HO
R-halide, TBAI,
NaH, DMF
RO
Nitration
(HNO3/HOAc)
O2N
RO
Reduction
H2 with Pd/C
H2N
RO
Sandmeyer reaction
1: HONO
2: MeOH
MeO
RO
H2 with Pd/C,
MeOH
OH
MeO
HO

Fig. 15

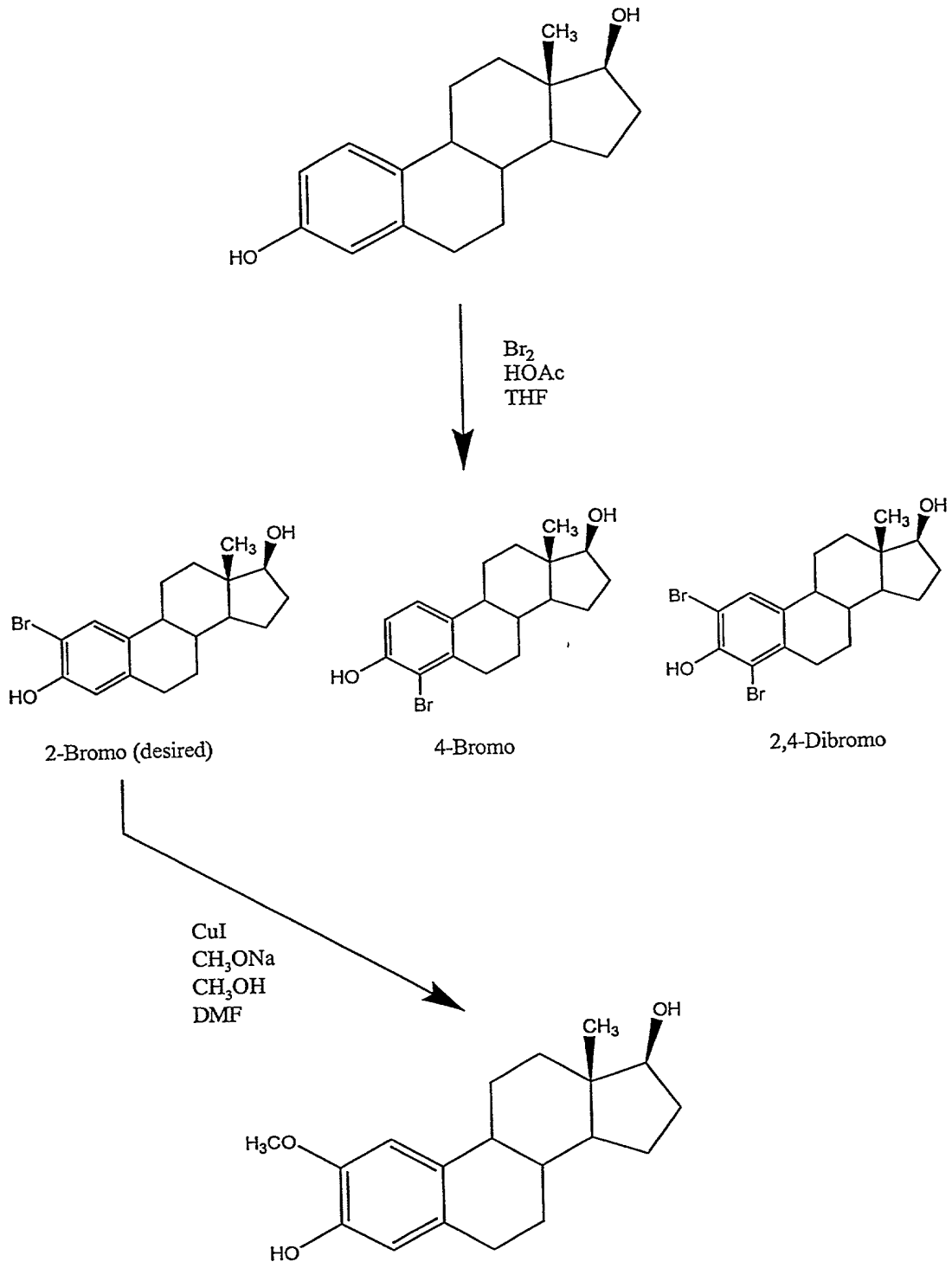

CH3
OH
HO
Br2
HOAc
THF
Br
HO
2-Bromo (desired)
4-Bromo
2,4-Dibromo
CuI
CH3ONa
CH3OH
DMF
H3CO

Fig. 16